IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: XU, K.

Application No.: 10/107,583

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Group: 1644

For: INOTROPIC ANTIBODIES AND THERAPEUTIC USES THEREOF

37 C.F.R § 1.132 DECLARATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Kai Y. Xu, PhD, declare as follows:

1. I am the sole inventor of the patent application No. 10/607,583 entitled "INOTROPIC ANTIBODIES AND THERAPEUTIC USES THEREOF " (hereafter the '583 application) and the subject matter described therein.
2. I hold a PhD degree in Chemistry and currently am working in Biomedical Science. I am presently an Associate Professor at the University of Maryland School of Medicine, Baltimore, Maryland.
3. Following submission of the patent application No. 10/607,583 on June 25, 2003, I have continued working on my invention as shown below as **Exhibit A.** The date of each figure only represents the date of summarizing the results and does

not represent the date of the individual experiment. The experimental results show that both Jianye-2 and KX-1 antibodies bind to the $(Na^+ + K^+)$-ATPase and enhance the activity of the enzyme. Please note that in the Exhibit A, Jianye-2 antibody is named as SSA78 and KX-1 is named SSA412.

4. **Exhibit A**

Exhibit A

Introduction

Under the experimental conditions described in the method, I discovered that Jianye-2 (SSA78)[1] and KX-1 (SSA412)[2,3] antibodies not only specifically bind to the SEQ ID NO: 1 and 2 sites located on the of the alpha subunit of the (Na^++K^+)-ATPase (NKA), respectively, but also accelerates the catalytic activity of the enzyme (Figures. 1, 2, & 3).

Method

Measurement of NKA activity

Monoclonal antibody SSA78 (mAb SSA78), polyclonal antibody SSA78 (pSSA78), and polyclonal SSA412 (SSA412) were used for the study. The enzymatic activity is defined as the ouabain-sensitive hydrolysis of MgATP in the presence of Na^+ and K^+. NKA activity is defined as ouabain-sensitive enzyme activity in different experiments. Purified ouabain-resistant rat NKA and ouabain-sensitive dog NKA were incubated with or without different concentrations of mAb SSA78 at 4 ^oC for 60 min. The reaction was initiated by adding MgATP (3 mM) in a final volume of 0.2 ml at 37 ^oC for 30 min and terminated by adding 0.75 ml quench solution (0.5% ammonium molybdate + 0.5 M H_2SO_4) and 0.02 ml developer (25 mg/ml of the mixture of 0.2g 1-amino-2-naphthol-4-sulfonic acid + 1.2 g sodium bisulfate + 1.2 g sodium sulfite). Color was developed for 30 minutes at room temperature and the concentration of phosphate was then determined at 700 nm using a spectrophotometer. In the purified enzyme system, incubation of NKA and specific antibody for 60 min prior to initiate ATPase assay is an optimal condition to obtain a significant activation of the enzyme.

Results

Result-1: Binding of mAb SSA78 to the H1-H2 Domain Increased NKA Activity.

Investigations of the effect of the interaction of mAb SSA78 at the H1-H2 domain of NKA was performed as described in the Method. Experimental results reveal that NKA activity is a function of the concentration of mAb SSA78 (Figure 1). Binding of mAb SSA78 to either ouabain-resistant rat NKA or ouabain-sensitive dog NKA increased the catalytic activity of the enzyme (Figure 1). The activity of ouabin-resistant rat NKA was 137±16, 148±21, 154±19, 162±14, 167±16, 174±13, and 182±10% in the presence of 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 2.0 mM mAb SSA78 compared with the control (in the absence of mAb SSA78) as shown in Figure 1A. Under the same experimental condition for the concentration of mAb SSA78, the activity of ouabain-sensitive dog NKA was 133±4.0, 142±11, 149±6.0, 157±5.0, 165±9.0, 174±12, and 176±17% (Figure 1B). The half effective concentrations (EC_{50}) for rat NKA and dog NKA were 0.141 and 0.154 μM, respectively (Figure 1). These data demonstrate a biological function of mAb SSA78 (Jianye-2) that is capable to increase the catalytic activity of NKA without inhibiting the enzyme function.

Result-2: Both mAb SSA78 and pSSA78 Increase Enzyme Activity by Interaction at the H1-H2 domain of NKA isolated from spontaneous hypertension heart failure (SHHF) rat. The effect of mAb SSA78 and pSSA78 on purified cardiac NKA that was isolated from heart failure rat model was further investigated. Figure 2 shows that by comparing with the control sample (without antibody), purified SHHF NKA activity was increased to 108±7.0, 125±23, and 187±22% in the presence of 1 μM mAb SSA78 (Figure 2, black circles), and 106±4.0, 121±12, and 205±16% with 1 μM pSSA78 (Figure 2, open triangles) during 10, 30, and 60 min interaction time course at the H1-H2 domain of the enzyme. No significant changes were observed in the control sample (Figure 2, open circles) and the samples in the presence of rabbit IgG (Figure 2, black triangles). No inhibition of SHHF NKA was detected in the presence of mAb SSA78 or pSSA78 (Figure 2).

Result-3: SSA412 Activates NKA and Increases the Activity of the Enzyme. Different concentrations of SSA412 (0.5 or 1.0 μM) were incubated with purified ouabain resistant- (rat) and sensitive- (dog) NKA prior to ATPase assay. Figure 3A & B shows that SSA412 markedly activated both rat and dog NKA function. The catalytic turnover of rat NKA was 34, 36, 42, 53, and 60 s^{-1} following 0, 10, 30, 60, and 80 min incubation with 0.5 μM SSA412 at 4 °C, and 34, 37, 48, 61, 72 s^{-1} with 1.0 μM SSA412 (Figure 3A). The same is true for the ouabain-sensitive dog NKA under the same experimental conditions as for rat NKA, the turnover of dog NKA was 53, 54, 58, 77, 86 s^{-1} with 0.5 μM SSA412, and 53, 55, 64, 85, 99 s^{-1} with 1.0 μM SSA412 (Figure 3B). In contrast, no significant changes in control samples (without SSA412) and in the presence of 1.0 μM total rabbit IgG for both rat and dog enzymes (Fig. 3A & B) were found indicating the specificity of SSA412-induced activation of NKA. These results indicate that NKA catalytic activity can be further accelerated in the presence of SSA412. To further verify this result, SSA412 was added to the samples after 60 min at room temperature (RT). In the absence of the enzyme substrate MgATP, the activity of purified NKA is gradually denatured at RT as a function of time as shown in Figure 3C & D (black circles). When partially inactivated NKA was exposed to SSA412, it remarkably protected both rat and dog enzyme functions by enhancing NKA catalytic turnover against further denaturing: rat NKA turnover was 16 s^{-1} (Figure 3C, black circles) following 60 min at RT before exposure to SSA412, and increased to 23 and 29 s^{-1} after incubation with 0.5 μM SSA412 for 30 and 60 min, respectively (Figure 3C, open circles). Under the same partially inactivation conditions, rat NKA turnover was further accelerated to 27 and 34 s^{-1} in the presence of 1.0 μM SSA412 (Figure 3C, black triangles). A similar phenomena was also observed for dog NKA: enzyme catalytic turnover was 32 s^{-1} at RT for 60 min (partial inhibition state at RT), and increased to 36 and 46 s^{-1} after exposure to 0.5 μM SSA412 for 30 and 60 min, and 41 and 52 s^{-1} with 1.0 μM SSA412 under the same experimental conditions.

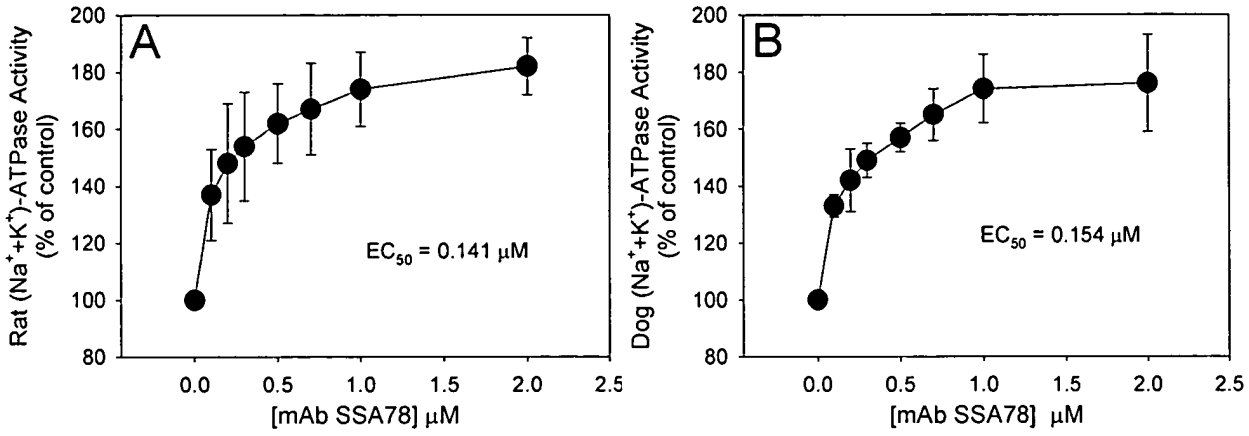

Figure 1. Effect of mAb SSA78 on the catalytic activity of NKA. Purified rat NKA (7.5 μg/ml) and dog NKA (1.3 μg/ml) were incubated with different concentrations of mAb SSA78 (as indicated in the figure) for 60 min at 4 °C in the presence of 100 mM Na^+ and 20 mM K^+ prior to ATPase assay. NKA activity significantly increased in the presence of mAb SSA78. Each data point represents the mean of four independent experiments.

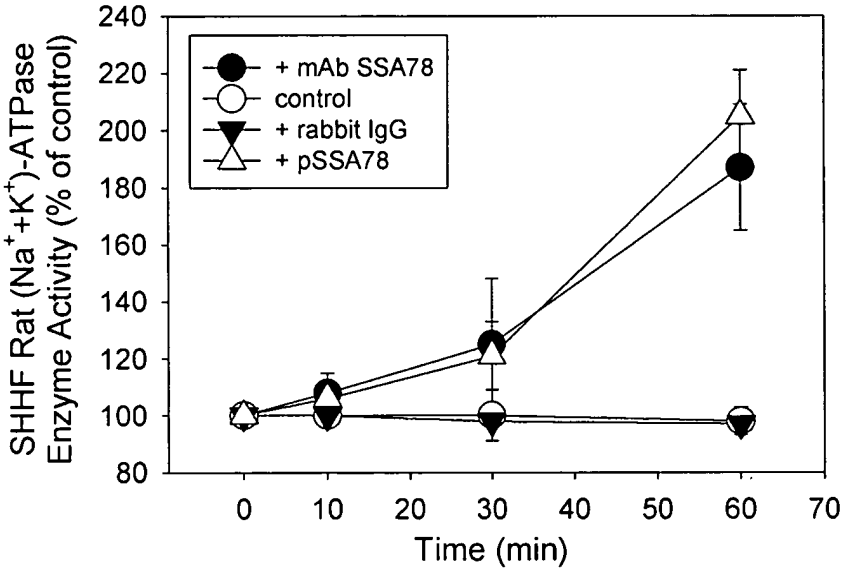

Figure. 2. Time course of the effect of mAb SSA78 and pSSA78 on SHHF NKA isolated from heart failure rat. Cardiac SHHF NKA (7.5 μg/ml) was incubated with or without mAb SSA78, or pSSA78, or total rabbit IgG (1 μM each) in different time course as indicated in the figure. Open circles: control; Black circles: with mAb SSA78; Open triangle: with pSSA78; Black triangle: with rabbit IgG. SHHF NKA activity was significantly increased in the presence of mAb SSA78 or pSSA78 after 60 min incubation. No changes of enzyme function were detected in the absence of both mAb SSA78 and pSSA78, or total rabbit IgG. Each data point represents the mean of three independent experiments.

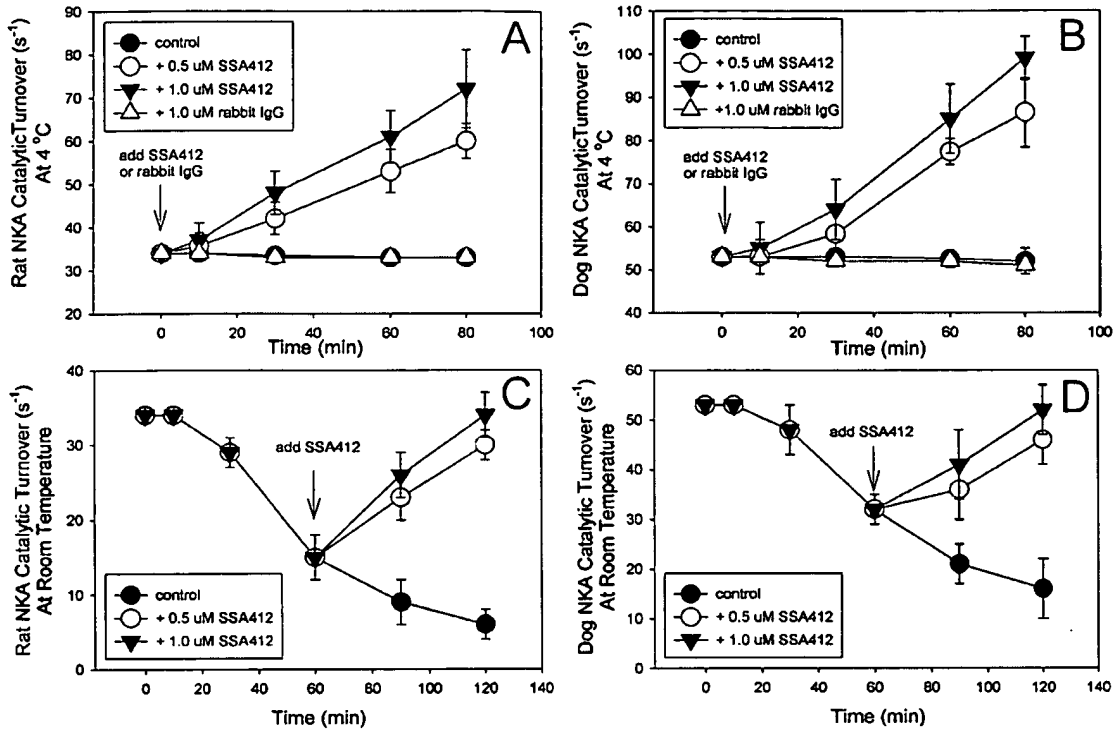

Figure 3. Time course of activation of both ouabain resistant- and sensitive- NKA. Purified rat (0.125 mg/ml, ouabain-resistant) or dog (0.125 mg/ml, ouabain-sensitive) NKA was incubated with SSA412 (0.5 and 1.0 μM) or with 1.0 μM normal rabbit total IgG at 4 °C (A, B) or at room temperature (C, D). NKA activity was monitored at 0, 10, 30, 60, and 80 min time point for experimental conditions A and B, and 0, 10, 30, 60, 90, 120 min for conditions C and D. The catalytic turnover number of NKA was calculated as indicated in the figure. The results show that binding of SSA412 to the enzyme accelerates the catalytic turnover number of both rat and dog NKA while activities of control (without SSA412 or with rabbit total IgG) remain the same at 4 °C (A, B). NKA turnover rate decreased at room temperature prior to adding SSA412 (C, D), SSA412 markedly protected the enzyme function by enhancing NKA catalytic turnover against further denaturing enzyme activity.

Summary

In summary, our experimental results provide direct evidence to demonstrate that both Jianye-2 and KX-1 antobodies are capable of regulating NKA function by increasing the catalytic activity of the enzyme.

Reference

1. Xu KY, Takimoto E, Juang GJ, Zhang Q, Rohde H, Myers AC. Evidence that the H1-H2 domain of α1 subunit of Na,K-ATPase Participates in the Regulation of Cardiac Contraction. FASEB (2005)19:53-61.
2. Xu KY. Activation of (Na^++K^+)-ATPase. Biochem Biophys Res Commun (2005)338:1669-1677.
3. Xu KY, Takimoto E, Fedarko NS (2006) Activation of (Na^++K^+)-ATPase induces positive inotropy in intact mouse heart in vivo. Biochem Biophys Res Commun 349:582-587.

5. I state that all experimental data provide herein are true under the experimental conditions including animal models used for the experiments. I further state that all statements made herein are true and that all statements made on information and experimental results under their experimental conditions are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

9/23/08

Date: _____

Kai Y. Xu

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Kai. Y. Xu